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Immunohistochemical p53 Protein Status in Nonsmall Cell Lung Cancer Is a Promising Indicator in Determining In Vitro Chemosensitivity to Some Anticancer Drugs

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Background and Objectives: The tumor suppressor oncogene p53 abnormalities have been closely associated with resistance or sensitivity of cancer cells to some chemotherapeutic agents. We examined the association between p53 protein status in nonsmall cell lung cancer (NSCLC) and in vitro chemosensitivity to several chemotherapeutic agents.

Methods: Using 146 surgically resected specimens of NSCLC, p53 status was immunohistochemically evaluated, and in vitro chemosensitivity to 5-fluorouracil (5-Fu), cisplatin (CDDP), mitomycin C (MMC), etoposide (VP-16), doxorubicin hydrochloride (ADM), and vindesine sulfate (VDS) was examined by a collagen gel-droplet embedded culture drug sensitivity test (CD-DST, Int J Oncol, 1997;11:449).

Results: Sixty-five of 146 materials (45%) showed immunohistochemically abnormal p53 protein accumulation in >10% of cancer cells within the tumor tissue, being regarded as p53+, whereas 81 (55%) were to p53−, in which no or less than 10% positive immunostaining cancer cells were detected. By CD-DST, the incidence of chemosensitive, borderline, and resistant p53− materials (N = 81) to 5-Fu was 37%(N = 30), 14%(N = 11), and 49%(N = 40), whereas that of p53+ materials (N = 65) was 20%(N = 13), 6%(N = 4), and 74%(N = 48), respectively, showing that p53− materials were significantly more sensitive to 5-Fu than p53+ materials ($P = 0.011$), especially in the adenocarcinoma type. As similar borderline association between p53 protein status and in vitro chemosensitivity was also shown in ADM ($P = 0.078$), but not in other chemoagents.

Conclusions: Immunohistochemically detected p53 protein status in NSCLC patients may be a promising indicator in determining in vitro chemosensitivity to some anticancer drugs, especially 5-Fu and ADM.

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KEY WORDS: **p53 protein; chemosensitivity; nonsmall cell lung cancer; collagen gel-droplet embedded culture drug sensitivity test; immunohistochemistry; 5-fluorouracil**

INTRODUCTION

The p53 tumor-suppressor gene encodes a 53-kD nuclear protein, which is involved in cell-cycle regulation. The wild type p53 protein may possess an antiproliferative and antitransforming activity [1-3] and, in some

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cases, may promote apoptotic death of cells through activation or suppression of transcription of some genes, e.g., GADD45 [4], the MDM2 gene [5], the WAF/CIP1 gene [6], and the Bax or Bcl-2 gene [7]. In contrast, inactivation of the p53 gene, which may be due mainly to point mutation or deletion of the gene, leading to a loss of function of the DNA binding and normal biological activity, may promote cyclin E/CDK2 kinase activity and induce a failure of cell arrest in G1 phase.

Recently, the p53 gene has been suggested to play an important role in transiently suppressing DNA synthesis and replication in cells when their DNA is damaged by chemotherapeutic drugs or radiation [8–10]. Cells lacking p53 function by mutation, continue to divide without effective repair of the DNA damage by anticancer agents and ionizing radiation and consequently do not take the programmed cell death pathway, suggesting that abnormalities of the *p53* gene are linked with the response to chemotherapeutic agents and radiation.

These alterations of the p53 protein are now considered to be a common event in human malignancies. In nonsmall cell lung cancer (NSCLC), abnormalities of the p53 gene are frequently encountered: p53 mutations are found at about a 50% rate by the polymerase chain reaction-single strand conformation polymorphism method [11,12]. Mutated p53 protein has a prolonged half-life compared with wild-type p53, and p53 protein accumulation in tumor cells correlates well with mutations of the p53 gene with a minor exception [13]. In fact, abnormal p53 protein accumulation is also found in 50% of NSCLCs [12].

Based on these findings, we analyzed the effect of the p53 gene abnormalities in patients with NSCLC with the sensitivity to chemotherapy. In particular, since immunohistochemistry for detection of p53 protein accumulation in tumor tissues is now rather easily and routinely performed for determining p53 abnormalities, we were very interested in the possibility of p53 protein as a marker of chemotherapeutic strategy. The present study was preliminarily conducted to determine the association between immunohistochemically detected p53 protein accumulation and the in vitro chemosensitivity of the surgically resected specimens of NSCLC to several chemotherapeutic agents, which are now commonly used for clinical patients.

MATERIALS AND METHODS

Specimens

The specimens were taken from 146 patients with NSCLC, who underwent surgery in our institute between June 1990 and July 1996. They included 107 men and 39 women; their ages ranged from 35 to 83 with a mean of 61.8. According to the international TNM staging system [14], 73 patients were in stage I, 17 were in stage II, 35 were in stage IIIA, 18 were in stage IIIB, and 3 were in stage IV, respectively. According to histological type, 38, 96, 9, and 3 patients had squamous cell carcinoma, adenocarcinoma, large cell carcinoma, and adenosquamous cell carcinoma, respectively. They had no preoperative cancer chemotherapy.

Immunohistochemical Detection of Abnormal p53 Protein Accumulation

Surgically resected formalin-fixed and paraffin-embedded tissue blocks of each patient, which had been well preserved at 4°C, were prepared for immunohistochemical analysis using the avidin-biotin-peroxidase technique to detect the abnormal p53 protein accumulation [12,15]. Briefly, sections (4 μm thick) from each block were deparaffinized, and endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol. Then, the sections were heated in a microwave oven for 2 × 5 min at 700 W in citrate buffer. After treatment with 2% normal horse serum, they were incubated with a monoclonal antibody, DO-7 (Novocastra Laboratories, Newcastle, UK), recognizing both mutant and wild forms of p53 protein. Sections were treated with biotinylated horse antimouse IgG (Vector, Burlingame, CA), and subsequently with an avidin-biotin peroxidase complex (Vectastatin ABC kit, Vector). The peroxidase reaction was processed with 0.02% diaminobenzidine and 0.01% hydrogen peroxide as chromogen. For each staining, a negative control was prepared by substituting the primary antibody with nonimmune mouse serum, and the positive control consisted of a lung squamous cell carcinoma known to exhibit nuclear p53 protein accumulation using the p53 antibody, DO-7.

For p53 protein immunostaining, only nuclear staining was considered. Nuclear staining was scored within the tissue, and the patients with >10% positive immunostaining cancer cells were regarded as p53+ (Fig. 1A), whereas those with no or <10% positive immunostaining cancer cells were regarded as p53− (Fig. 1B) [12]. Currently, it is accepted that a cutoff point of 10% positive immunostaining cancer cells is the most reliable for determining p53 abnormalities [12].

In Vitro Chemosensitivity Test

In vitro chemosensitivity was examined by the method of collagen gel-droplet embedded culture drug sensitivity test (CD-DST), described previously with minor modifications [16–18]. Briefly, surgically resected specimens were freshly minced using a scalpel and digested at 37°C for 1 h in dispersion collagenase enzyme, 0.1% EZ® (Nitta Gelatin, Yao, Japan). The dispersed cancer cells were collected by centrifugation, suspended in PCM-1 medium™ (Nitta Gelatin), and incubated in a collagen gel coated-flask, CG-flask™ (Nitta Gelatin) in a CO2 incubator at 37°C for 24 h. The viable cells alone adher-

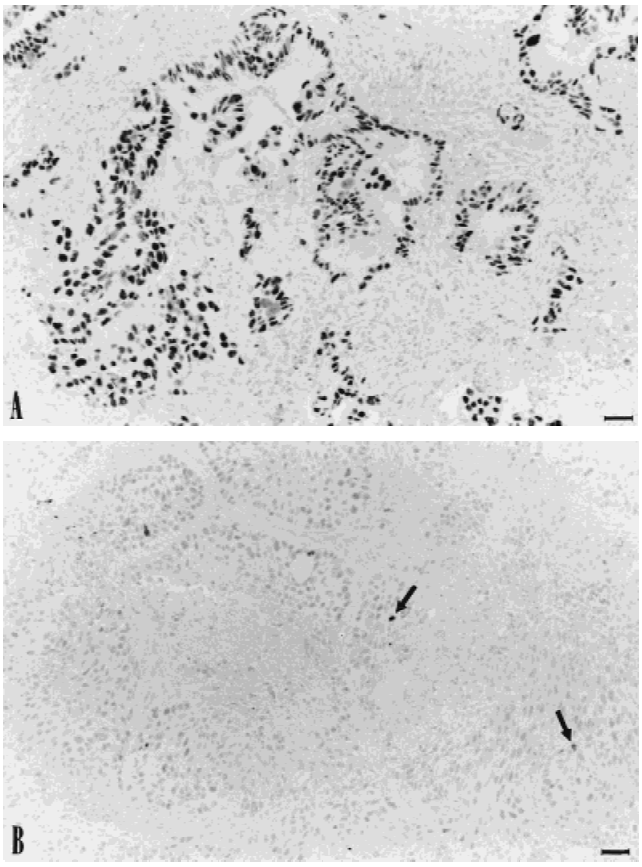

Fig. 1. **A.** Example of positive immunohistochemical stain indicating p53 protein accumulation in p53+ lung adenocarcinoma tissue. Note the intense nuclear staining evident in almost tumor cells (original magnification, ×40). Bar = 38μm. **B.** Example of immunohistochemical stain indicating no p53 protein accumulation in p53− lung squamous cell carcinoma tissue. Note the nuclear staining evident only in <10% tumor cells within the tumor tissue (arrow), whereas almost tumor cells show no immunostaining (original magnification, ×40). Bar = 38μm.

ing to the collagen gel-layer were collected after dissolving collagen gel-layer by 0.05% enzyme EZ™.

The cells prepared above were then added into the reconstructed Type I collagen solution (Cell matrix® Type CD™, Nitta Gelatin) to a final density of 1×10^5 cells/ml. Three drops of such mixtures (30 μl/drop) were placed in each well of a 6-well multiplate and allowed to gelate in a CO2 incubator at 37°C for 1 h, after DF medium™ (Nissui Pharmaceutical, Tokyo, Japan) with 10% fetal bovine serum (FBS, Gibco, Gaitherberg, MD) was overlaid on each well.

The anticancer agents were added to each well to give a final concentration of 1.0 μg/ml for 5-fluorouracil (5-Fu, Kyowa Hakko Kogyo Co., Tokyo, Japan), 1.0 μg/ml for etoposide (VP-16, Nippon Kayaku Co., Tokyo, Japan), 0.02 μg/ml for doxorubicin hydrochloride (ADM, Kyowa Hakko Kogyo Co., Tokyo, Japan), 0.01 μg/ml for vindesine sulfate (VDS, Shionogi & Co., Osaka, Japan), 0.2 μg/ml for cisplatin (CDDP, Bristol-Myers Squibb Inc., Tokyo Japan), and 0.03 μg/ml for mitomycin C (MMC, Kyowa Hakko Kogyo Co., Tokyo, Japan), and then incubated for 24 h. After removal of the medium containing the anticancer agents, each well was incubated with PCM-2 medium™ (Nitta Gelatin) for 7 d. Then, neutral red (50 μg/ml) was added to each well to stain colonies in the collagen gel droplets. Such droplets were finally fixed with formalin buffer.

The total volume of cancer cell colonies was estimated by the image analysis system (VIDAS Plus, Carl Zeiss, Germany).

The in vitro cytotoxic effect of each anticancer agent was expressed as a ratio of the total colony volume (T) of the treated cells to that of the untreated cells (C). The sample with a ratio of T to C of 50% or less, >60%, and between 50 and 60%, was judged as sensitive, resistant, and borderline, respectively.

Statistical Analysis

Differences were evaluated by the Chi-square tests, and the *P* values were from one-tailed tests; $P<0.05$ and $0.05 \leqslant P< 0.10$ were considered to be statistically significant and marginally significant, respectively.

RESULTS

Of the 146 tested materials, 81 (55%) were p53−, and 65 (45%) were p53+. The incidence of chemosensitive, borderline, and chemoresistant materials to each anticancer agent was; 43 (29%), 15 (10%) and 88 (60%) for 5-Fu, 43 (29%), 13 (9%) and 90 (62%) for ADM, 46 (32%), 15 (10%) and 85 (58%) for VP16, 46 (32%), 15 (10%) and 85 (58%) for VDS, 44 (30%), 16 (11%) and 86 (59%) for CDDP, and 46 (32%), 9 (6%) and 91 (62%) for MMC.

Association between p53 protein accumulation status and in vitro chemosensitivity is shown in Table I. The cases with p53− were more sensitive to 5-Fu compared with those with p53+ with a statistically significant difference ($P=0.011$): the incidence of sensitive, borderline, and resistant p53− cases (N = 81) was 37%(N = 30), 14%(N = 11), and 49%(N = 40), whereas that of p53+ cases (N = 65) was 20%(N = 13), 6%(N = 4), and 74%(N = 48), respectively. Similarly, those with p53− showed higher sensitivity to ADM with a marginally significant difference ($P=0.078$): the incidence in 81 specimens of sensitive, borderline, and resistant p53− cases was 37%(N = 30), 7%(N = 6), and 56%(N = 45), whereas that of p53+ cases (N = 65) was 20%(N = 13), 11%(N = 7), and 69%(N = 45), respectively. The p53− samples also appeared to have higher sensitivity to VP-16 without a statistically significant difference ($P=0.102$): the incidence of sensitive, borderline, and resistant p53− cases in 81 specimens was 38%(N = 31), 11%(N = 9), and 51%(N = 41), whereas that of p53+ cases was 23%(N = 15), 9%(N = 6), and 68%(N = 44),

TABLE I. Association Between p53 Protein Status and In Vitro Chemosensitivity by CD-DST in 146 NSCLC Patients

Chemoagent		In Vitro Chemosensitivity			
	No. of	Sensitive	Borderline	Resistant	
p53 status	patients	No. of patients (%)			*P* value
5-FU					0.011
p53−	81	30(37)	11(14)	40(49)	
p53+	65	13(20)	4(6)	48(74)	
ADM					0.078
p53−	81	30(37)	6(7)	45(56)	
p53+	65	13(20)	7(11)	45(69)	
VP16					0.102
p53−	81	31(38)	9(11)	41(51)	
p53+	65	15(23)	6(9)	44(68)	
VDS					0.229
p53−	81	27(33)	11(14)	43(53)	
p53+	65	19(29)	4(6)	42(65)	
CDDP					0.829
p53−	81	26(32)	9(11)	46(57)	
p53+	65	18(28)	7(11)	40(62)	
MMC					0.197
p53−	81	28(35)	7(9)	46(57)	
p53+	65	18(28)	2(3)	45(69)	

respectively. There was no association between the chemosensitivity to the other agents and p53 protein status in the tested materials (VDS; $P = 0.229$, CDDP; $P = 0.829$, MMC; $P = 0.197$).

Since a significant association between chemosensitivity to 5-Fu and p53 protein status was observed in NSCLC ($P = 0.011$), further analysis was performed concerning the chemosensitivity and histological type (Table II). In 96 patients with adenocarcinoma, p53− showed significantly better in vitro chemosensitivity to 5-Fu than p53+ ($P = 0.011$): the incidence of sensitive, borderline, and resistant p53− cases (N = 59) was 37%(N = 22), 15%(N = 9) and 47%(N = 28), whereas that of p53+ cases (N = 37) was 16%(N = 6), 5%(N = 2), and 78%(N = 29), respectively, but such an association was not observed in 38 squamous cell carcinoma patients ($P = 0.486$).

TABLE II. Association Between p53 Protein Status and In Vitro Chemosensitivity to 5-FU by CD-DST According to Histological Type

Histology		In Vitro Chemosensitivity			
	No. of	Sensitive	Borderline	Resistant	
p53 status	patients	No. of patients (%)			*P* value
Adenocarcinoma (n = 96)					0.011
p53−	59	22(37)	9(15)	28(47)	
p53+	37	6(16)	2(5)	29(78)	
Squamous cell carcinoma (n = 38)					0.486
p53−	14	4(29)	2(14)	8(57)	
p53+	24	6(25)	1(4)	17(71)	

DISCUSSION

In the present study using surgically resected specimens of NSCLC patients, we surveyed immunohistochemical p53 protein status and in vitro chemosensitivity to several representative chemotherapeutic agents, showing a close association with some chemotherapeutic agents: NSCLCs with p53− were more sensitive to 5-Fu than those with p53+ with a statistically significant difference ($P = 0.011$), and this result was remarkably observed in the cases with adenocarcinoma ($P = 0.011$). Also, a similar borderline association was shown in ADM ($P = 0.078$). Thus, p53 abnormalities in NSCLCs may play a crucial role in modulating drug-induced cytotoxicity in 5-Fu and ADM.

In malignant tumors, several analyses to determine an association between p53 abnormalities and chemosensitivity to some anticancer agents have been reported. Experimentally, Lowe et al. [19] described that wild-type p53-tumors showed a better response to ADM treatment or radiation, compared to those with mutant type p53 or p53-deficient type, in an in vivo assay system using fibrosarcoma cell line. Koechli et al. [20] showed an association of mutation of p53 in breast cancer with in vitro CMF chemoresistance (CMF = cyclophosphamide, methotrexate, and 5-Fu), and a similar result was also obtained in lymphoma cells [21] and brain tumor [22]. Clinically, p53 abnormalities have been also regarded as a possible indicator of chemoresistance in colorectal [23], ovarian [24], and breast cancers [25]. In contrast, there was no association between p53 abnormalities and chemotherapeutic effect on the response rate and survival rate of patients with cancers of the esophagus [26] and breast [27] and rather conflicting results in esophageal cancer by Muro et al. [28].

In the previous reports using lung cancer cell lines, no such association was observed [29,30], but this conclusion may be unacceptable because of the small number and high incidence of p53 abnormalities in their tested materials. In contrast, NSCLC patients with p53 abnormalities showed a lower response to chemotherapeutic agents, compared to those without p53 abnormalities [15,31]. Furthermore, Fujiwara et al. [32] reported that adenovirus-mediated transfer of the wild-type p53 gene in the NSCLC cell line increased chemosensitivity. In the present study, in which the number of materials was sufficiently large, p53 abnormalities in NSCLC might be closely associated with sensitivity to some chemotherapeutic agents, although it was only based on an in vitro assay.

Interestingly, of the six anticancer agents examined in this study, a significant association was shown only in 5-Fu. In ADM, borderline association was also shown. This association of p53 abnormalities and chemosensitivity to 5-Fu and ADM in other malignancies was previously described in an in vitro or in vivo assay [10,19,33]. Also clinically, chemotherapy with a combi-

nation of anticancer drugs including 5-Fu was more effective on wild-type p53-patients with colorectal [23] and breast cancers [25]. In NSCLC, the chemotherapeutic effect of 5-Fu drug has been controversial, but postoperative adjuvant chemotherapy using UFT, a combined anticancer drug of tegafur (FT) and uracil at a molar ratio of 1:4, has recently been reported to be significantly effective for prolonged survival [34]. Such an effect of UFT chemodrug may be partially concerned with p53 status in NSCLC patients.

Currently, CDDP-based chemotherapy is regarded as a standard therapy on NSCLCs. As shown by some studies [15,31], p53 status in NSCLC patients may be useful in determining the cellular response to chemotherapy with CDDP. Further, the gene therapy studies by Roth's group with cell lines has also shown a convincing relationship between p53 status and CDDP responsiveness [32,35]. In the present study, however, there was apparently no association between p53 abnormalities and in vitro chemosensitivity to CDDP. To this discrepancy, since the current results of p53 abnormalities in NSCLCs were based only on immunohistochemical analysis, the p53+ materials in our series might possibly show a wide mutational spectrum of the p53 gene, and such cases with p53− homozygously deficiency type as NSCLC cell lines, H358 and H1299 [32,35], possibly might be included in the p53− group in this study. Moreover, it has been generally accepted that NSCLCs with p53-homozygously deficiency type are rather unusual. Therefore, at least, immunohistochemically detected p53 protein status apparently may not be an indicator for chemosensitivity to CDDP, namely, CDDP-induced apoptosis in NSCLCs. Wu et al. [36] showed a close association between p53 abnormalities and chemosensitivity to VP-16 in lung cancer cell lines, but this association was not reproduced in our series, although its *P* value was 0.102. Thus further analysis may be required in these problems.

Various in vitro chemosensitivity tests for malignant tumors have been described, but in contrast to small cell lung cancers, their clinical usefulness remains controversial in NSCLCs [37,38]. CD-DST, first established by Koezuka et al. [16], is now considered to be a reliable technique to provide information of chemosensitivity to anticancer drugs [17,18], but there are several clinical disadvantages: specimens must be taken in the culture system without contamination, and this system may take at least 2 wk, and is expensive. In this respect, immunohistochemistry for detection of p53 protein will be routinely and easily performed. In fact, some investigators [15,26,31] described that p53 status by immunohistochemistry using biopsy specimens before treatment may be a practically useful indicator of chemotherapeutic response. Therefore, immunohistochemical analysis of p53 protein may provide an important information for the clinical application of a regimen including 5-Fu for NSCLC patients, in particular, the cases with adenocarcinoma, even if an in vitro chemosensitivity test was not available.

In lung cancers, there are several useful indicators including neuroendocrine markers [39], P-glycoprotein [40], topoisomerase II [41], multidrug resistance-associated protein (MRP) [42,43], glutathion S-transferase-π (GST-π) [44], erb-B2 oncoprotein [30], and Bcl-2 protein [45,46], which may be associated with chemosensitivity to some anticancer agents, and interestingly, some of them may be clinically applied as a prognostic factor in association with therapeutic effect [39,44]. This study showed that immunohistochemically abnormal p53 protein accumulation in NSCLC, especially in the adenocarcinoma type, is also predictive of in vitro chemoresistance to 5-Fu and ADM. Therefore, p53 protein status may be a promising indicator in determining a clinical strategy for chemotherapy using such anticancer drugs for the patients with this disease. Further study is warranted in assessing the association between p53 protein status and therapeutic efficacy.

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